DRUG-PLASMA BINDING MEASURED BY SEPHADEX*

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A method is described using the cross-linked dextran gel, Sephadex, for the study of drug binding. Although not quantitative in terms of per cent bound, this method gives an indication of the comparative strength of binding. The usefulness of this method in the separation of compounds with different plasma binding characteristics is suggested.

The availability of the cross-linked dextran gel, Sephadex, provides a promising system for the *in vitro* study of drug binding. Porath and Flodin (1959) first described its use and pointed out the possibility of separating molecules of different size by a method they refer to as "gel filtration". This cross-linked hydrophilic dextran polymer expands upon hydration but remains particulate and its high degree of cross linkage gives rise to a gel grain of low porosity which permits only molecules of small size to enter the internal volume of the grains. Molecules of larger size, such as protein, are excluded from the grains and appear in the early eluate of chromatographic columns. This principle has been used to separate bound and unbound fluorescein (Zwaan and Van Dam, 1961).

In the course of studies on the entry and accumulation of a number of isotopically labelled compounds in brain, we have investigated drug binding in plasma and tissue. Binding in plasma is important in assessing the relative quantity of a drug that is available for passage into the brain and cerebrospinal fluid. In addition, a number of our observations on regional or diffuse brain accumulation appears to be related to drug-tissue binding (Roth and Barlow, 1961). The nature of the binding of drugs in plasma and brain is unknown and the significance of this phenomenon is under study in various laboratories where the techniques of ultrafiltration, dialysis, differential centrifugation and electrophoresis have been employed in *in vitro* systems. This paper presents a method for the study of drug binding in plasma utilising the cross-linked dextran polymer, Sephadex.

Materials and Methods

Carbon-14 labelled phenytoin, thiopentone, phenobarbitone and barbitone were synthesised by Mr. L. Clark in the radiochemical laboratory of the Department of Pharmacology at the University of Chicago. These labelled drugs were shown by paper chromatography in multiple systems to be chemically and radiochemically pure. ^{14}C urea was purchased from Volk Radiochemical Company, Chicago, and sodium ^{35}S sulphate was purchased from Abbott Laboratories, Chicago. Normal human plasma was obtained from the blood bank of the University of Chicago and

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diluted to the desired concentration with 0·1M phosphate buffer of pH7·4. Sephadex G-25 was purchased from Pharmacia, Uppsala, Sweden.

Five g. dry gel were hydrated in 0·1M phosphate buffer pH 7·4, allowed to settle and the "fines" decanted. The hydrated Sephadex was then poured into a column 3 cm. in diameter and allowed to settle. A filter paper disc was placed on the top of the gel. Columns to be eluted with plasma were washed with 50 ml. of plasma just before application of the sample. Columns so prepared allowed a free flow of eluting fluid and could be used repeatedly.

One ml. of buffer or plasma containing drug and naphthol blue black dye as a protein indicator was placed on the column. When the sample had entered the column it was washed on by 1 ml. of elution fluid. The elution fluid (buffer or plasma) was then added in quantity and 3 ml. fractions were collected. One ml. aliquots of the radioactive eluates were plated on copper planchettes and counted in a Packard gas flow Geiger-Mueller counter. Protein was assayed colorimetrically (Gornall, Bardswill and David, 1949) and naphthol blue black was measured at 640 mμ in a Coleman Junior Spectrophotometer (Bailey and Heald, 1961).

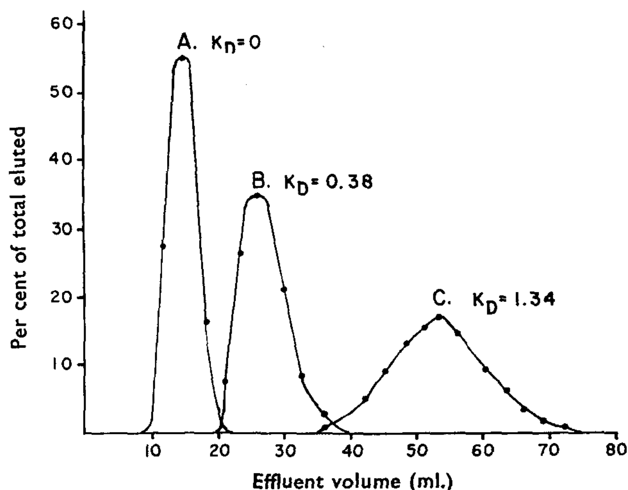

FIG. 1. Each point represents the per cent of total test substance eluted. Curve A represents the elution pattern of plasma protein eluted with 0·1M phosphate buffer pH 7·4 ($K_D = 0$). Curve B represents the elution pattern of phenytoin eluted with 50 per cent plasma, pH 7·4 ($K_D = 0{\cdot}38$). Curve C represents the elution pattern of phenytoin eluted with phosphate buffer 0·1M, pH 7·4 ($K_D = 1{\cdot}34$). One ml. of sample was applied in each case and the same column was used.

The partition ratio between the two aqueous phases in a Sephadex column is represented by the K_D value (Gelotte, 1960).

$$K_D = \frac{Ve - Vo}{Vi}$$

Vo is the water outside the gel grains, or "outer volume", while Vi is the water within the gel grains, or the "inner volume". Vi, the inner

volume, is calculated from the "water regain"* value (Granath and Flodin, 1961). Vo, the outer volume, is determined for each run and is the volume of eluate required to deliver the protein or protein dye complex in peak concentrations. Ve is the volume of eluate required to deliver the peak concentration of the experimental substance. When Ve and Vo are equal, as is the case if the test substance is of a molecular weight which restricts it to the outer volume, the K_D value is 0. A substance of low molecular weight which can diffuse freely into the grains and occupies both the inner and outer volumes will have a K_D value of 1. In practice, low molecular weight substances have a K_D value of 0·8 to 1·0, apparently because some of the water of hydration of the gel is bound (Gelotte, 1960). A K_D value greater than 1 indicates adsorption to the gel (Gelotte, 1960). The relationship between K_D values and effluent volume is represented in Fig. 1.

Results

In Table I, data are presented demonstrating that naphthol blue black (0·25 mg./ml. plasma) was eluted in the external volume, i.e., ($K_D = 0$) when the elution fluid was either phosphate buffer or plasma. If the dye,

TABLE I

Gel filtration of naphthol blue black in PO_4 buffer and plasma

Eluent	Naphthol blue black	K_D
PO_4 buffer pH 7·4 ..	0·25 mg. in 1 ml. plasma	0
Plasma 50 per cent ..	0·25 mg. in 1 ml. plasma	0
PO_4 buffer pH 7·4 ..	0·25 mg. in 1 ml. PO_4 buffer	4·12
PO_4 buffer pH 7·4 ..	0·5 mg. in 1 ml. 10 per cent plasma	peak I = 0 peak II = 4·17
Plasma 50 per cent ..	0·5 mg. in 1 ml. plasma	0

TABLE II

Gel filtration of drugs in PO_4 buffer and plasma

	Plasma concentration mg./ml.	Eluent: PO_4 buffer pH 7·4			Eluent: 50 per cent plasma		
		K_D*	Range	Runs	K_D*	Range	Runs
Phenytoin	0·01	1·22	(1·03–1·34)	5	0·35	(0·31–0·41)	6
Thiopentone	0·05	1·15	(1·15–1·15)	3	0·36	(0·32–0·38)	3
Phenobarbitone ..	0·05	1·30	(1·21–1·38)	4	1·02	(1·02–1·02)	2
Barbitone	0·05	0·84	(0·72–0·93)	4	0·94	(0·86–1·00)	4
Urea	Trace and 0·5	0·93	(0·83–1·00)	5	0·92	(0·90–0·93)	4
Sulphate	Trace and 0·5	0·83	(0·76–0·93)	7	0·92	(0·90–0·93)	5

* See text.

dissolved in buffer, was applied to the column and eluted with buffer then a K_D value of 4·12 was obtained indicating that the dye had been adsorbed on the gel. If an excess of dye (0·5 mg./ml. of 10 per cent plasma) was applied to the column, the dye was then eluted in 2 peaks by the buffer. One peak was associated with the plasma protein ($K_D = 0$) and the second peak was eluted at K_D 4·17, the value obtained with dye alone.

* Supplied by manufacturer with each batch.

As can be seen from Table II, phenytoin applied in plasma, was eluted at K_D 1·22 when phosphate buffer was the eluent indicating minimal binding to the gel. Similar K_D values were obtained when the drug was applied dissolved in buffer. When plasma was used as the eluent, however, the K_D of phenytoin was shifted toward the K_D of substances restricted to the external volume phase (K_D 0·35). Unlike the dye, phenytoin was never eluted at $K_D = 0$. Table II also shows that thiopentone behaved similarly. Phenobarbitone followed the same general trend, but the decrease in K_D with plasma as eluent was less marked. In experiments with phenytoin 10 per cent plasma eluted the drug with a K_D of 0·71, 25 per cent plasma with a K_D of 0·58, as compared to the K_D of 0·35 when 50 per cent plasma was employed. The use of undiluted plasma gave K_D values in the same range as 50 per cent plasma.

The K_D values for urea, barbitone and sulphate were similar whether these substances were eluted with buffer or plasma. These data indicate that these substances are not bound by plasma, a finding consistent with previous studies utilising other methods. In addition, these data serve as controls for possible changes in the gel related to the presence of plasma which could lead to a change in K_D unrelated to drug-plasma binding.

Discussion

Before discussing the results of this study, it is useful to consider the results obtained by accepted techniques. The plasma binding properties of the four drugs we have investigated here have been determined by ultrafiltration through a Cellophane membrane by the method of Lavietes (1937) or *in vivo* by comparison of the cerebrospinal fluid and plasma levels at equilibrium. By these methods, phenytoin and thiopentone were found by us (unpublished) to bind to plasma protein to the greatest extent (77 per cent), phenobarbitone to a lesser extent (37·7 per cent) (Domek, Barlow and Roth, 1960), while barbitone was found by others to exhibit little if any tendency to bind to plasma protein (2 per cent) (Brodie, Kurz and Schanker, 1960). Using ultrafiltration we have found that sulphate ion and urea remain unbound. Ultrafiltration of plasma containing up to 0·5 mg./ml. naphthol blue black yielded no dye in the ultrafiltrate. However, when an aqueous solution of the dye (0·5 mg./ml.) was subjected to ultrafiltration, we found a considerable fraction was adsorbed on the Cellophane resulting in 22 per cent recovery in the ultrafiltrate (data to be published). Clearly, ultrafiltration through a Cellophane membrane is not suitable to study binding of this dye and an error due to adsorption is introduced. This source of error should be considered whenever a membrane is utilised to study binding.

In the Sephadex system one might expect that the portion of test substance associated with protein molecules would be excluded from the gel grains and eluted in the early fraction ($K_D = 0$). The free or unbound drug should appear in a second elution fraction representing the internal volume ($K_D = 0{\cdot}8$–$1{\cdot}0$), or with a K_D greater than 1·0 if it is adsorbed to the gel. This situation applies when naphthol blue black is added

in excess as illustrated in Table I, where 0·5 mg. dye in 1 ml. 10 per cent plasma was eluted by buffer in 2 peaks. The first peak was associated with the protein and had a K_D value of 0 while the second had a K_D of 4·17, comparable to the dye alone. On the other hand, we were unable to saturate the phenytoin and thiopentone systems which were eluted as one fraction whether these drugs were applied in concentrations of pharmacologic interest or in great excess (2 mg./ml.), and whether they were placed on the column dissolved in plasma or buffer. However, the volume of eluent required to recover these drugs was found to be dependent upon the protein content of the eluent. When eluted by phosphate buffer they were recovered with a K_D value of approximately 1·2. When 50 per cent plasma was employed, they were brought down at $K_D = 0{\cdot}35$. Phenobarbitone demonstrated a similar change, but to a lesser degree, while the elution volume of barbitone, urea and sulphate was unaltered regardless of the eluent. We conclude that binding to the plasma protein in the eluent was responsible for the reduction in the K_D of phenytoin, thiopentone and phenobarbitone. This change in K_D may therefore be used as a qualitative measure of binding. Barbitone, sulphate and urea, known not to bind appreciably to plasma protein, were unaffected by the protein concentration of the eluent and served as controls for nonspecific changes related to the composition of the eluting fluid and the gel itself.

The contrast between the plasma-dye interaction and the plasma drug interaction is of interest. The strength of the binding with the dye was such that naphthol blue black remained with the plasma in which it was originally dissolved as it passed down the column. The drugs studied did not do this, but apparently dissociated from the protein of the plasma in which they were dissolved. This difference between the dye and the drugs indicates a difference in the strength of binding to protein. If plasma protein was present in the eluting fluid, the presumed adsorption of the drugs to the gel was overcome and phenytoin, thiopentone and phenobarbitone were eluted with a K_D value distinctly lower than that found when buffer was employed as the eluent. Naphthol blue black was more firmly bound and was eluted at a K_D of zero with either buffer or plasma eluent.

Gel filtration with Sephadex is a useful addition to the procedures available for the study of drug binding *in vitro* because it avoids the use of an interposed potentially adsorbing membrane and gives some indication of strength of binding. It may be adaptable to the study of binding of drugs to various constituents of tissues such as brain by using eluents containing tissue components. Gel filtration with plasma eluent may also serve as a method for separation of mixtures of compounds which differ in the degree of binding to plasma; for example, barbitone from thiopentone, phenobarbitone from phenytoin.

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